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Comparative Genotoxicity of Herbicide Ingredients Glyphosate and Atrazine on Root Meristem of Buckwheat (*Fagopyrum esculentum* Moench)

Girjesh.Kumar and Akanksha Srivastava*

Plant Genetics Laboratory, Department of Botany, University of Allahabad-211002 India

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Abstract

Herbicides have been extensively used in agriculture but people have not concerned about its adverse effects on plants and animals. The aim of the present study is to analyze the detrimental effects of two herbicides in *Fagopyrum esculentum* Moench (Variety: VL-7). The root tips were treated with four concentrations viz. 50, 100, 150 and 200 ppm of glyphosate and atrazine at room temperature for 3 hrs. Mitotic indices and chromosomal anomalies were calculated. It was observed that both the herbicides induced different types of chromosomal abnormalities comprising of scattering, precocious movement, stickiness, bridges, laggard etc. along with the increasing doses of herbicides. Scattering and stickiness are most prevalent abnormalities among others. The effect of glyphosate was more toxic than atrazine in the root meristem of *Fagopyrum esculentum*.

Keywords: *Fagopyrum esculentum* Moench, Herbicides, Chromosomal anomalies, Mitodepressive, Mitotic indices.

1. Introduction

Herbicides have been extensively used for a better exploitation of different plants in modern agriculture and landscape turf management. They are effective but have a strong biological activity against plants. Across the world, they are manufactured to cultivators who suffered heavy losses due to weed. However, the advantages come at a significant cost. They are persistent to degradation, and get bio accumulated in the environment affecting higher organisms and producing undesirable secondary consequences in plants. They can also be classified by their "site of action" or the specific biochemical site that is affected by the herbicides. Their properties increase the likelihood of transport including resistance to degradation and high water solubility.

One of the most widely commercialized herbicides in the world is glyphosate (Vivancos 2011). It is categorized under the systemic EPSPS (Enzyme-5 enolpyruvyl-shikimate-3-phosphate synthase) inhibitor herbicides inactivated by soil content that can control most annual and perennial plants. EPSPS is an enzyme which is found only in plants and micro-organisms. It controls the weeds by inhibiting the synthesis of aromatic amino acids such as tryptophan and tyrosine which is necessary for protein formation in susceptible plants (Pipke *et al.*,1987). It also affects other biochemical processes and although these effects are considered, they may be important in the total lethal action of glyphosate.

Atrazine is categorized under triazine class used to prevent pre and post emergence broadleaf weeds in variety of crops such as maize, sorghum, sugarcane and some extent in landscape vegetation. In the United States as of 2014, atrazine was the second most widely used herbicides after glyphosate found in the rural environment. It does not occur naturally. It is prepared from cyanuric chloride when treated sequentially with ethylamine and isopropyl amine. Plants can absorb atrazine through roots or through the foliage. Once absorbed, it is accumulated in the growing tips and the new leaves of the plant, inhibiting photosynthesis in susceptible plant species.

Many studies have been done to study the effect of the herbicides including cyanazine, gespax, goltix, aventox and atrazine on the mitotic activity, chromosomes and nucleic acids content in root tip cells of different plants in a large scale. (Wuu and Grant, 1966; Liang *et al.*, 1967; Stroev, 1970; Hakeem and Shehab, 1972; Liang and Liang, 1972; Dryanovska and Petkov, 1980 Badr, 1983, 1986; Badr *et al.*, 1985; Mousa, 1982a, Tomaskova and Mydilova, 1986; Airapetyan *et al.*, 1984; Papes *et al.*, 1989; Haliem, 1990; Ashour and Abdou, 1990). The previous authors stated that all the herbicides inhibited the cell division and produced chromosomal abnormalities and in some cases the inhibition was integrated with the reduction of the nucleic acids.

Buckwheat is a pseudocereal belongs to Polygonaceae family and its diploid chromosome no. is 16. But the

* Corresponding author. e-mail: srivas.akanksha20@gmail.com.

chromosome sizes are small, which makes the cytogenetical analyses complicated (Neethirajan *et al*., 2011). It is a very easily grown plant; it prefers dry sandy soils but succeeds in most conditions including poor, heavy or acid soils and even sub-soils. It is frequently cultivated for its edible seeds and leaves. It is very nutritious food. The primary production of pseudocereal is its seeds which have relatively higher contents of carbohydrate and calories as compared to other cereal and it is also most desirable crop because all parts of plants are valuable such as seeds and leaves used for medicinal purposes, mainly in diabetes, celiac disease and gluten allergies. It is a good honey plant producing a dark, strong monofloral honey. It is easily contaminated with herbicides from the environment because of its medicinal use; during growth and rejuvenation process when its readymade products are produced. Testing of plant roots are very useful because root meristem is the place for exposure of chemicals which spread in environment, soil and water.

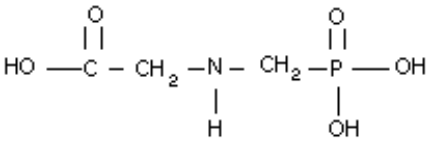
It is therefore of interest to conduct the effect of glyphosate and atrazine on cell division, chromosome behaviour and DNA and RNA contents in the root tip cells of *Fagopyrum esculentum* in turn responsible for mutagenecity and its outcome may help in understanding the possible constraints in the role of these two herbicides as toxic in plants.

2. Material and Method

For the cytological study, the seeds of buckwheat variety VL-7 were collected from National Bureau of Plant Genetics Resources, Shimla, Phagli, India. For the mitotic studies, seeds were germinated in petridish into an incubator for 1-2 days at 22-25^{0}C.

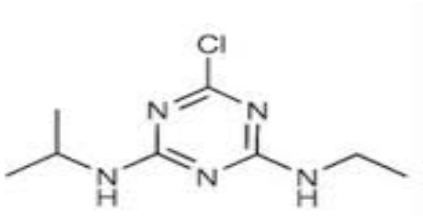
2.1. Herbicide compounds

1. Glyphosate- Molecular formula- $C_3H_8NO_5P$
(IUPAC Name: N- (phosphonomethyl) glycine**)**
Structural formula-



Molecular weight- 169.07 g·mol^{-1} (Health & Consumer Protection Directorate-General. 2002) Active purity percentage- > 80%

2. Atrazine- Molecular formula-$C_8H_{14}ClN_5$
(IUPAC Name: 6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine**)** (World Health Organization 2011) Structural formula-



Molecular weight- 215.68 g·mol^{-1} (Plakas *et al*., 2006)

2.2. Herbicides treatment

After the emergence of root tips, different concentrations of glyphosate and atrazine solutions were prepared by using dilution method. Then, the seeds were treated by soaking method with different concentrations viz. 50,100,150 and 200 ppm of each herbicides for 3 hrs and one set of germinated seeds were also maintained as control with distilled water only. The root tips were then washed thoroughly in distilled water and fixed in carnoy's fixative (1 Glacial acetic acid: 3 alcohol) for 24 hours and were preserved in 90% ethanol.

2.3. Cytological analysis

The root tips were hydrolysed in 1N HCl for 2-3 minutes for softening and then stained with 2% acetocarmine for 30 minutes. Finally, slides were prepared by using squash technique for cytology and observation was done at 40X resolution in light microscope and photographs were taken by using PCTV Vision Photography Software.

Following the formulae used in the calculation of Active mitotic index and abnormality percentage –

$$\text{Active mitotic index (AMI) \%} = \frac{\text{Total no. of dividing cell}}{\text{Total no. of cell observed}} \times 100$$

$$\text{Total Abnormality percentage (TAP) \%} = \frac{\text{No. of Abnormal cell}}{\text{Total no. of cell observed}} \times 100$$

2.4. Statistical Analysis

Statistical analysis was performed using the SPSS 16.0 software. Three replicates for each treatment and one independent variable were used. A one way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT, $P < 0.05$) were performed for mean separation and the graph was plotted by using sigma plot 10.0 software. Actual mean and standard error were calculated. The data were subjected to analysis of variance.

3. Results

In buckwheat plant, the eight basic (Morris, 1951) chromosome number has been found (2n=2x=16). The cytological observation revealed that the mitotic index in control sets was found to be 12.62%, with normal chromosome behaviour, showing perfectly normal arrangement of 16 chromosome at metaphasic plate (Figure1A) and 16:16 separation during anaphase (Figure1B). However, after the treatment of the root tip cells with glyphosate and atrazine changes in normal behaviour of chromosome and rate of dividing cells were

observed. Mitotic indices and chromosomal abnormality percentages of both the herbicides treatment sets were inversely related to each other.

The ranges of Active Mitotic Index (AMI) and chromosomal aberrations (Total Abnormality Percentage-TAP) with respect to the doses of herbicides have been summarized in Table 1. Figure 2 shows a decline in the value of active mitotic indices along with the increasing concentration of herbicides, i.e., glyphosate and atrazine. In case of glyphosate treated set, the percentage of active mitotic index reduced from 10.98% to 4.64%, whereas in case of atrazine, it was reduced from 11.85% to 6.90% on increasing the concentration from 50 to 200 ppm. It has been investigated that a lower dose was not much effective for the plant tissues and abnormalities might be recovered but the highest dose, i.e., 200 ppm, severely damaged the plant cells and also destroyed the cells, causing a cell elongation and a cell distortion, So, the maximum abnormality percentage and the lowest active mitotic indices were observed at higher doses of treatment in both cases viz. 200 ppm glyphosate (TAB% 9.24, AMI% 4.64) and atrazine (TAB% 7.05, AMI% 6.90) shown in Table 1. Both the herbicides induced various types of chromosomal aberrations (Figure1) such as scattering, c-mitosis, precocious movement, unorientation, stickiness, laggard with forward movement, bridge, etc. But the glyphosate induced a higher percentage of chromosomal abnormalities as compared to atrazine (Figure 3).

Most of the aberrations were present predominantly at higher doses of treatment, eg., scattering, stickiness and unorientation were found at the highest doses (200ppm) treatment set of glyphosate and atrazine respectively (Table 1).

The total abnormalities increased from 3.52% to 9.24% and 2.73% to 7.05% as the doses increased from 50 ppm to 200 ppm in treated sets of glyphosate and atrazine, respectively.

Table 1. Abnormality induced by Glyphosate & Atrazine in root meristems of *Fagopyrum esculentum* Moench

Treatment	Doses (ppm)	AMI % (Mean ± SE*)	Metaphasic abnormalities (%) (Mean ± SE)						Anaphasic abnormalities (%) (Mean ± SE)				TAP (%) (Mean ± S.E)
			Cm	Sc	St	Pr	Un	Br	St	Un	Lg	Oth	
Glyphosate (*N*-(phosphonomethyl)glycine)	Control	12.62± 0.02^{a**}	--	--	--	-	--	--	--	--	--	--	--
	50	10.98± 0.04^{b}	0.36± 0.01^{a}	0.61± 0.12^{b}	0.36± 0.01^{b}	0.55± 0.17^{b}	0.37± 0.01^{a}	0.55± 0.19^{a}	0.55± 0.19^{a}	0.48± 0.11^{b}	0.37± 0.11^{b}	0.36± 0.01^{a}	3.52± 0.32d
	100	9.04± 0.27^{c}	0.52± 0.15^{a}	0.68± 0.01^{b}	0.46± 0.10^{b}	0.81± 0.23^{ab}	0.46± 0.11^{a}	0.46± 0.10^{a}	0.70± 0.22^{a}	0.69± 0.20^{b}	0.81± 0.11^{a}	0.35± 0.01^{a}	5.44± 0.14^{c}
	150	6.52± 0.25^{d}	0.59± 0.17^{a}	0.89± 0.12^{b}	0.69± 0.25^{ab}	0.69± 0.08^{ab}	0.79± 0.11^{a}	0.39± 0.09^{a}	1.09± 0.12^{a}	1.19± 0.16^{a}	0.79± 0.83^{a}	0.49± 0.09^{a}	7.63± 0.24^{b}
	200	4.64± 0.22^{e}	0.64± 0.07^{a}	1.45± 0.11^{a}	1.05± 0.07^{a}	1.13± 0.05^{a}	0.89± 0.22^{a}	0.49± 0.23^{a}	0.98± 0.25^{a}	1.38± 0.10^{a}	0.97± 0.03^{a}	0.38± 0.14^{a}	9.24± 0.15^{a}
Atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine)	Control	12.62± 0.02^{a}	--	--	--	--	--	--	--	--	--	--	--
	50	11.85± 0.14^{b}	0.35± 0.15^{b}	0.47± 0.01^{b}	0.35± 0.02^{a}	0.36± 0.10^{a}	0.37± 0.01^{a}	0.35± 0.15^{a}	0.35± 0.16^{c}	0.37± 0.01^{b}	0.35± 0.15^{a}	0.37± 0.01^{a}	2.73± 0.11^{d}
	100	10.97± 0.03^{c}	0.35± 0.06^{b}	0.47± 0.12^{b}	0.59± 0.12^{a}	0.47± 0.11^{a}	0.47± 0.12^{a}	0.35± 0.01^{a}	0.70± 0.07^{b}	0.58± 0.11^{b}	0.47± 0.12^{a}	0.58± 0.11^{a}	4.79± 0.14^{c}
	150	8.93± 0.09^{d}	0.52± 0.17^{ab}	0.57± 0.11^{b}	0.46± 0.12^{a}	0.87± 0.19^{a}	0.57± 0.11^{a}	0.45± 0.10^{a}	0.79± 0.09^{b}	1.13± 0.87^{a}	0.57± 0.11^{a}	0.46± 0.12^{a}	5.93± 0.19^{b}
	200	6.90± 0.15^{e}	0.72± 0.25^{a}	1.07± 0.19^{a}	0.71± 0.19^{a}	0.72± 0.21^{a}	0.71± 0.19^{a}	0.48± 0.13^{a}	1.32± 0.12^{a}	0.96± 0.13^{a}	0.37± 0.01^{a}	0.53± 0.16^{a}	7.05± 0.14^{a}

Where:Cm- C- Mitosis, Sc- Scattering, St- Stickiness, Pr- Precocious movement, Un- Unorientation, Br- Bridge, Lg- Laggard, Oth- Others *SE= Standard error **Means followed by lowercase letter are statistically significant at $p < 0.05$ in Duncan multiple range test.

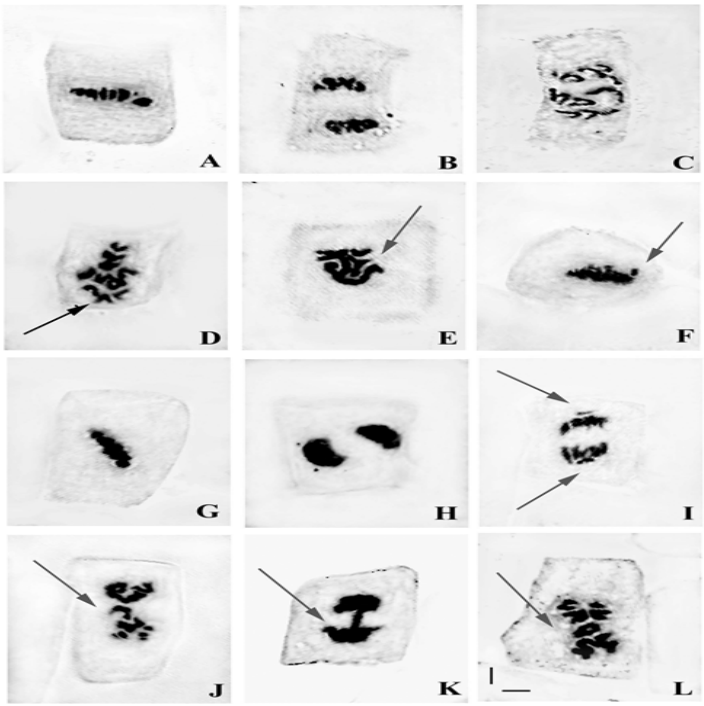


Figure 1. **A**.Normal Metaphase (2n =16);**B.**Normal Anaphase (16:16 separation);**C.** Scattering ;**D**.C- mitosis;E.Clumping at Metaphase;**F**.Precocious movement;**G**.Unorientation with stickiness at Metaphase;H**.**Unorientation with stickiness at Anaphase;I.Forward movement at Anaphase;**J**.Laggard formation;**K.**Single bridge at Anaphase;**L**.Disturbed polarity at Anaphase .
[Bar length = 6.42µm, width = 7.5 µm]

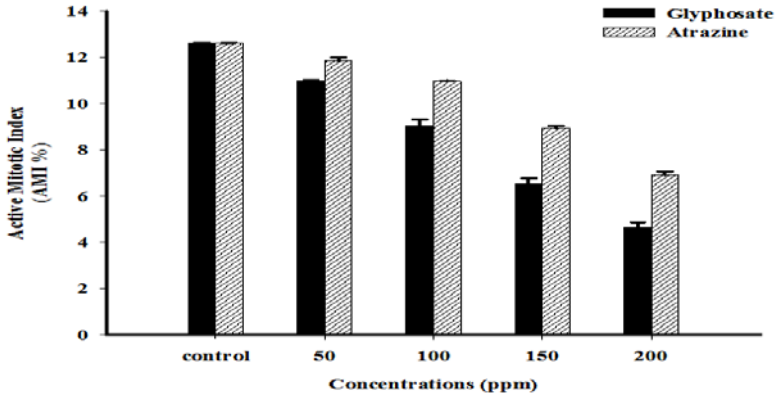


Figure 2. Comparative account of Active Mitotic Index (AMI) for Glyphosate and Atrazine in the root meristem of *Fagopyrum esculentum* Moench

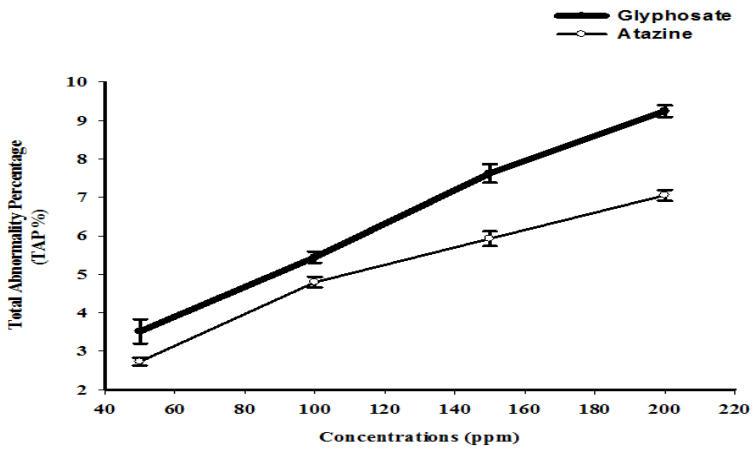


Figure 3. Comparative account of Total abnormality percentage for Glyphosate and Atrazine in the root meristem of *Fagopyrum esculentum* Moench

4. Discussion

The mitotic index refers to the frequency of cell division which is an important parameter for determining the rate of root growth (Liu *et al.*, 1992). The results of the present study show that there is a gradual reduction in mitotic index as a result of the increase in the herbicides concentration. This gradual reduction of mitotic index and higher rate of chromosomal abnormalities clearly show that herbicides are toxic for plant at chromosome level. Chemicals which affect the plants and induce chromosome damaging are called as clastogens and their action on chromosome is generally regarded to involve an action on DNA (Grant, 1978; Chauhan *et al.*,1990). In the current findings, scattering, stickiness and unorientation were most frequent abnormality after treating the root tip cells with glyphosate and atrazine, respectively. The higher concentration, i.e., 200 ppm, was effective in inducing a statistically significant increase in the frequency of abnormal mitosis. But the toxicity of glyphosate is more than atrazine which is clearly proved at cytological level.

The highest frequency of scattering and spindle dysfunctioning were recorded at 200 ppm of glyphosate and the reason was attributed to the loss of microtubules of the spindle fibres and also shows co-relation with the precocious c-mitosis types of chromosome anomalies (Figure 1 C). Another reason for the formation of precocious chromosome is due to early terminalisation of the chromosome or due to chemical breakage of the protein moiety of nucleoprotein backbone (Patnaik, 1984) (Figure 1 F).

Cytotoxic and mitodepressive effects of glyphosate and atrazine, used in the present case, were in conformity with the earlier findings. (Saxena *et al*., 2004; Gul *et al.*, 2006; Mustofa and Arikan, 2008). C-mitosis was the most frequent chromosomal abnormality observed during metaphase at 150 and 200ppm in both the treated sets (Table 1, Figure 1 D). Arrested metaphasic cells indicated that the herbicide in the present investigation cause disturbance or inhibition of spindle formation similar to the effect of colchicines and lagging chromosome (Figure 1 J) were the result of impairment of mitotic apparatus, treatment of 100 and 200 ppm in the treatment of glyphosate gave a higher frequency of laggards (Haliem, 1990; Sonia Sharma and Adarsh Pal Vig, 2012).

Chromosomal Stickiness was very frequent in both herbicides treatment at the 200 ppm (Figure 1G and H) and it is induced either by the effect of herbicides on the chromosomal protein which is attributed to the irregular folding of chromosome fibres or due to the action of herbicides on the polymerization process, resulting in the fragmentation of chromosome bridges forms sticky chromosomes (El- Gha-Mery *et al*., 2000). It might have occurred due to the sudden contraction of some of the spindle fibres using to toxic effect of glyphosate. (Mahakhode and Somkuwar, 2013).

Chromosome fragmentation is also observed in the root meristem of *Fagopyrum esculentum* due to these herbicides treatment, which results from the breaks of chromosome in which there is a loss of chromosome integrity. Amer and Ali (1969) also reported that Pentachlorophenol induced fragmentation of both mitotic and meiotic chromosome of *Vicia faba* (Grant, 1978).

Chromosome bridges were not observed in root tip cells of both the treatments with the lowest concentration where an abundance of breakage and clumping was recorded. The proportion of bridges generally increased on increasing concentration, i.e., at the highest dose viz. 200 ppm. It is formed due to the sticky behaviour of chromosomes which could not move towards pole regions at anaphase (Kumar and Rai, 2007) or due to the chromosomal stickiness and subsequent failure of free anaphasic separation or inversion of chromosome (Najjar and Soliman, 1980). The breakage of chromosomes at the same locus and their lateral fusion led to the formation of dicentric chromosome. It plays a main role in the bridge formation (Figure 1 K). It was pulled equally towards both the pole at anaphase and bridges were formed (Anis *et al.*, 1998).

Such chromosomal abnormalities may affect adversely the vigour, fertility and yield of exposed plant. Herbicides with such action can also alter the genetic constitution of crop, resulting in mutational change which could be very dangerous. Hence, the highest concentration of both herbicides may become genotoxic, chromotoxic and clastogenic for crop plants in the environment. Therefore, its higher concentration is not suggestive to all people especially the herbicide glyphosate because its toxicity is too high.

5. Conclusion

From the above foregoing discussion, the results obtained in the present study indicate cytotoxic activity of glyphosate was more as compared to atrazine on the basis of cytological study. So, if a higher concentration of herbicides is present in the environment and absorbed by the plants, it may adversely affect the genetic system causing damage to the chromosome in crop plants. Regular uses of herbicides in agricultural practices are a potential threat to the genetic constitution of crop plants and animals. Therefore, judicial uses of these herbicides are essential. An indiscriminate use of herbicide should be discouraged as far as practicable. Rather, it should be replaced with bio-herbicides and bio- control agents which do not pose adverse risks to crops as well as the ecosystem.

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